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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of : Knipe, et al.  
Serial No. : 08/278,601  
Filed : July 21, 1994  
For : Herpesvirus Replication Defective Mutants  
Group : 1645  
Examiner : Caputa, A.

Assistant Commissioner of Patents  
Washington, D.C. 20231

**DECLARATION UNDER 37 C.F.R. §1.608(b)**

I, David Knipe, declare:

1. That I am the sole inventor of the subject matter set forth in Appendix A. My curriculum vitae is attached hereto as Appendix B.
2. That Robert Finberg and George Siber were named as co-inventors in this application as a result of their inventive contribution with respect to a composition and method for treating an immunomodulatory disease by use of a mutated herpesvirus that has an ability to effect an antibody subclass shift.
3. That the subject matter of Appendix A was originally described in U.S. Application Serial No. 07/922,912, filed July 31, 1992, now abandoned, in which I was named as sole inventor.
4. That the subject matter of Appendix A was conceived and reduced to practice in the United States prior to September 25, 1990.

5. That the subject matter of Appendix A was reduced to practice at my request and on my behalf in the United States prior to September 25, 1990 by demonstrating that two different mutant herpesviruses protected mice against a lethal dose of wild-type herpesvirus, HSV-mP. The mutant herpesviruses that provided such protection were not capable of producing additional virus in cells other than cells that complemented the defective genes. In particular, the mutated viruses used in these experiments consisted of one herpesvirus containing a deletion mutation in the gene that expresses ICP8, known as mutant d301; and the other herpesvirus containing a nonsense insertion mutation in the gene expressing ICP27, known as mutant n504R.

6. That to the best of my information and belief, Appendices C, D and E attached hereto are true copies, with dates deleted, of laboratory notebook pages written in conjunction with the performance of experiments performed at my request and on my behalf in the United States before September 25, 1990.

7. That the experiments performed at my request and on my behalf in the United States prior to September 25, 1990 were as follows.

8. That an ICP27 gene nonsense codon insertion mutant herpesvirus n504R that was replication defective was produced.

A. An ICP27 gene nonsense codon insertion mutant herpesvirus n504R was propagated and titrated on V-27 cells. V-27 cells can produce the mutant herpesvirus because they contain an integrated copy of the ICP27 gene, thus they complement the growth of HSV-1 ICP27 mutants and serve as hosts for the isolation of ICP27 mutants. A replication defective mutant such as n504R will not however replicate on normal cells such as Vero cells which are routinely used for growth of HSV-1, since they lack an ICP27 gene.

B. V-27 cells were infected with an n504R mutant herpesvirus. After harvesting the propagated herpesvirus from the cells, the n504R mutant herpesvirus was aliquoted and frozen at  $-70$  and designated "M28 n504R". A sample of the virus was tested for its ability to replicate by measuring plaque formation in cells infected with the mutant herpesvirus. The mutant herpesvirus n504R was tested for plaque formation on V-27 cells and on Vero cells. The results showed that the n504 mutant herpesvirus failed to produce plaques on the Vero cells at the lowest dilution ( $10^{-3}$ ) that could be read (lower dilutions killed the monolayer). At that same low dilution the V-27 cell monolayer had too many plaques to count. Distinct and measurable plaques were formed on the V-27 cells at much higher dilutions tested in the V-27 cells ( $10^{-7}$ ). A virus titer of  $4 \times 10^8$  was calculated. These results confirmed that n504R is a replication defective ICP27 mutant herpesvirus.

9. That the following correlates the experiment described in paragraph 8 to the notebook pages provided in Appendix C:

A. The notebook page records the making of a stock of n504.a mutant herpesvirus. This is stated on the second line of the page:

"For each stock:.....n504R".

B. Cells that had been infected and incubated were harvested, frozen and thawed twice and sonicated. The harvested virus was aliquoted, tubes labeled and frozen.

This is stated on the first notebook page at the third line:

"Shake off.

Into 2 200 ml bottle.

10', 1.5K

Resuspend in 20 ml                      10 ml supe + 10 ml milk.

Freeze @ -70

Thaw 2x

Sonicate 3x30" @ setting 4.

Aliquot to 40 vials.

"M28 n504R".

C. Three days later, the virus was tested at a series of dilutions ( $10^{-1}$  to  $10^{-8}$ ) for plaque formation on V27 and Vero cells. There was no plaque formation in the Vero cells and positive plaque formation in the V-27 cells. The titer of the mutant virus was calculated to be  $4 \times 10^8$ . This is stated on the notebook second page:

"	-1	-2	-3	-4	-5	-6	-7	-8
n504R-V27			>>	>>	>>	>>	31	2
n504R-Vero	k	k	0	0	0	0		

k= killed monolayer

Titers for muts	V27:	Vero
n504R	$4 \times 10^8$	$< 1 \times 10^3$

10. That an ICP8 gene internal deletion mutant herpes virus d301 that was replication defective was produced.

A. An ICP8 gene internal deletion mutant herpesvirus d301 was propagated and titrated on S-2 cells. S-2 cells can produce the mutant herpesvirus because they express the ICP8 protein from a resident gene upon viral infection, thus they provide the complementing gene product that the mutant protein is missing. A replication defective mutant such as d301 will not however replicate on normal cells such as Vero cells which are routinely used for growth of HSV-1, since they lack an ICP8 gene.

B. S-2 cells were infected with d301 mutant herpesvirus. After harvesting the propagated virus from the cells, the virus was tested for its ability to replicate by measuring plaque formation in cells infected with the mutant herpesvirus. The mutant herpesvirus ICP8 d301 was tested for plaque formation on S-2 cells and on Vero cells. The results showed that the d301 mutant herpesvirus failed to produce plaques on the Vero cells, even at the lowest dilution tested ( $10^{-2}$ ). At that same low dilution the S-2 cell monolayer was destroyed by the d301 mutant

herpesvirus. At much higher dilutions ( $10^{-7}$ ) distinct plaques were formed on the S-2 cells, were counted and a viral titer of  $1.7 \times 10^9$  was calculated. These results confirmed that d301 is a replication defective ICP8 mutant herpesvirus. The d301 mutant herpesvirus was aliquoted and designated "d301.a \_\_\_\_\_" stock.

11. That the following correlates the experiment described in paragraph 10 to the notebook page provided in Appendix D:

A. The notebook page records the making of a stock of d301.a mutant herpesvirus. This is stated on the top of the page:

"Stock of d301.a"

B. Four T-150 tissue culture flasks were each seeded with  $10^7$  S-2 cells, passage 14.  $4 \times 10^5$  pfu of d301A-1, having a titer of  $1.2 \times 10^7$  was added to each T-150 flask. The cells and virus were incubated for 1 hour at  $37^\circ\text{C}$ , the virus was removed and the cells were incubated at  $37^\circ\text{C}$ . This is stated on the notebook page at the top:

"①	4-T-150	S-2	p14	$10^7$ cells/T-150
②	d301A-1	$1.2 \times 10^7$	_____	
③	0.01 pfu	x 4 flasks	x $10^7$ =	$4 \times 10^5$ "
	"10 ml/T-150	$37^\circ\text{C}$	/	hr
	Suck off	add 45 ml	$37^\circ\text{C}$ "	

C. The infected cells were harvested, resuspended, sonicated and the virus-containing suspension was aliquoted. A sample of the propagated mutant herpesvirus was tested for plaque formation. There was no plaque formation in the Vero cells and positive plaque formation in the S-2 cells. The titer of the mutant herpesvirus was  $1.7 \times 10^9$ .

This is stated on the notebook middle of the page:

" \_\_\_\_\_ AM Harvest add 12 ml supn, 6 ml milk  
 ....sonicated aliquoted  
 "d301.a \_\_\_\_\_"  
  

	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>
vero	0	0		
S-2 p17			85	6

  

$$85 \times 2 \times 10^7 = 1.7 \times 10^9$$

12. That an aliquot of the n504R stock described in paragraph 8 was delivered to the laboratory of Robert Finberg prior to September 25, 1990.

13. That an aliquot of the d301a stock described in paragraph 10 was delivered to the laboratory of Robert Finberg prior to September 25, 1990.

14. That the replication defective mutant herpesviruses n504R and d301 provided a protective immune response in animals to challenge with wild type herpesvirus.

A. 10<sup>6</sup> pfu of replication-defective viruses, those containing mutations in the genes encoding ICP8 or ICP27, were injected into mice, and then challenged with a lethal dose of 10<sup>8</sup> pfu live wild-type HSV-1 virus. The mice that received the mutants had 100 % survival rates whereas the control mice that did not receive mutant virus had a 10 % survival rate. Thus the experiments demonstrated that replication defective mutants of HSV-1 induced immunity in mice injected with the mutant viruses and protected against lethal infection whereas the majority of mice injected with control material and subsequently challenged with wild type virus, died.

15. That the following correlates the experiment described in paragraph 14 to the notebook pages provided in Appendix E:

A. Female Balb/c mice, 5 to 7 weeks of age, were used for the experiment. These mice were injected intraperitoneally with the viruses or control samples.

This is stated on page **HOO3388**:

second line "injection mice with";

third line right side of page near the margin "n=8 Balb";

just below the middle of the page on the right across from the number ②  
"n=8Balb mice".

and on page **HOO3497** first and second lines where it is written

"① Balb mice....♀. 5-7 weeks by \_\_\_\_\_."

"n" refers to the number of mice in the group.

B. Viruses used in the experiment were obtained from the laboratory of Dr. David Knipe. The mutant viruses were ICP8 stock d301 and ICP27 stock n504R.

This is stated on page **HOO3388**

top line on the left "All virus received from Dr. David Knipe \_\_\_\_\_";

fifth line: "ICP8 stock d301 \_\_\_\_\_ received from Dr. David Knipe's Lab (Kay)  
on day \_\_\_\_\_."

8<sup>th</sup> line following ②:"ICP27 (n504R)".

The titer of the viruses was  $1.7 \times 10^9$  pfu/cc for the ICP8 mutant virus page **HOO3388** 8th line and  $4 \times 10^8$  pfu for ICP237 (n504R) on line midpage in paragraph ②.

"ICP8" refers to the replication defective mutant virus containing a mutation in the gene encoding ICP8, termed d301; "ICP27" refers to the replication defective mutant virus containing a mutation in the ICP27 gene, termed n504R.

C. The mutant viruses were diluted in PBS to  $10^6$  pfu/cc in an injection volume per mouse of .5cc.

This is shown on page **HOO3388**:

par.① 6<sup>th</sup> line "Need  $10^6$  pfu/cc. so do a 1:1700 dilution that means :100 $\lambda$  in 170000 = 170cc or: 100 $\lambda$  (virus stock) in 85cc PBS and injection of .5cc"

par.② 2<sup>nd</sup> line "Need  $10^6$  pfu/cc: So do a 1: 4  $10^2$  Dilution that means 100 $\lambda$  in 40000 = 40cc. PBS or 100 $\lambda$  (virus) in 20cc and inject 0.5cc."

D. Groups of eight mice were injected with  $10^6$  pfu of each of the replication defective mutants ICP8 (d301) and ICP27 (n504R). Control mice (group of 9 mice) were injected with PBS.

This is shown on page **HOO3388**:

third line right side of page near the margin "n=8 Balb";

just below the middle of the page on the right across from the number ②  
"n=8Balb mice".

and on page **HOO3497**

par. ① "Balb mice.... ♀. 5-7 weeks by \_\_\_\_\_."

" \_\_\_\_\_ : challenged with

106pfu HSV ICP8 n=8 (2)

106pfu HSV ICP27 n=8 (3)

and n=9 (4)

PBS as control"

E. Six weeks plus 5 days later, all the mice were challenged with  $10^8$  pfu of a virulent wild-type HSV-1 strain , HSV-1 (mP).



This is shown on notebook page **HOO3497** mid page:

"Date deleted : challenge with  $10^8$  pfu HSV-mP."

F. Mortality was determined 10 days post challenge with HSV-1mP. As reported on notebook page HOO3487, two mice each from the groups injected with mutant virus were removed for proliferation assay studies leaving six mice per group. The mice which had been inoculated with the mutants ICP8 (d301) or ICP27 (n504), 0 (zero) mice of six died; 1/9 control (PBS injected) mice survived, that is 8 out 9 mice died.

This is shown on the bottom half of page **HOO3497** as follows:

"Mortality:

in 10 days

(2)	(ICP8)	0 died.
(3)	(ICP27)	0 died.
(4)	8 died from 9.	(control)"

16. That the work described in paragraphs 8 - 11 was performed in my laboratory.

17. That the work described in paragraphs 14 and 15 was performed in the laboratory of Robert Finberg, at my request, with mutant herpesviruses produced in my laboratory.

18. That I hereby declare that all statements made herein are true, and all statements made on information and belief are believed to be true, and further that all statements were made with the knowledge that any willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Date: September 4, 1998

David M. Knipe  
David Knipe

## APPENDIX A

A vaccine comprising a pharmaceutically acceptable excipient and an effective immunizing amount of a mutant herpesvirus, said mutant herpesvirus containing a genome in which a viral gene encoding a protein which is essential for production of infectious virus has been deleted or inactivated, wherein said mutant virus is able to cause production of infectious new virus particles in a recombinant complementing host cell expressing a gene which complements said essential vital gene, but is unable to cause production of infectious new virus particles when said mutant virus infects a host cell other than said recombinant complementing host cell, for prophylactic or therapeutic use in generating an immune response in a subject infected therewith.



APPENDIX B

updated 7/98

CURRICULUM VITAE

Name: David Mahan Knipe

Title: Higgins Professor of Microbiology  
and Molecular Genetics,  
Harvard Medical School

Date of Birth: August 6, 1950

Place of Birth: Lancaster, Ohio

Social Security Number: 269-50-2351

Marital Status: Married, two children

Home Address: 58 Auburn St.  
Auburndale, MA 02166

Home Phone No: (617) 969-7239

Laboratory Address: Dept. of Microbiology and  
Molecular Genetics  
Harvard Medical School  
200 Longwood Avenue  
Boston, MA 02115

Office Phone No: (617) 432-1934

Telefax number: (617) 432-0223

Electronic mail: dknipe@warren.med.harvard.edu

Education:

Case Western Reserve University, B.A. in Biology, summa cum laude, 1972.

Massachusetts Institute of Technology, Ph.D. in Cell Biology, 1976. Thesis research on vesicular stomatitis virus with Dr. David Baltimore and Dr. Harvey Lodish.

University of Chicago, post-doctoral training, 1976-1979, with Dr. Bernard Roizman on molecular genetics of herpes simplex virus.

#### Positions held:

##### Academic and Professional

Assistant Professor of Microbiology and Molecular Genetics, Harvard Medical School, 1979-1984.

Assistant Chairman, Committee on Virology, Harvard University, 1982-1985.

Associate Professor of Microbiology and Molecular Genetics, Harvard Medical School, 1984-1989.

Chairman, Committee on Virology, Harvard University, 1985-1991.

Program Director, NIH Training grant on "Mechanisms of Viral Infectivity," 1986-present.

Professor of Microbiology and Molecular Genetics, Harvard Medical School, 1989-1997.

Director, Tridepartment Graduate Program, Harvard Medical School, 1991-1994.

Teaching Coordinator, Department of Microbiology and Molecular Genetics, 1996-present.

Higgins Professor of Microbiology and Molecular Genetics, Harvard Medical School, 1997-present.

##### Consulting

Consultant, Merck, Sharp and Dohme Research Laboratories, 1983-1986.

Consultant, Gene-Trak, 1990-1991.

Consultant, Hybridon, Inc., 1991-1992, 1993-1994.

Consultant, Eli Lilly, Inc., 1991-1992.

Consultant, OraVax, 1991.

Consultant, Virus Research Institute, Inc., 1995-present.

### Editorial

Associate Editor, *Virology*, B. Fields, editor in chief; R.M. Chanock, J.L. Melnick, B. Roizman and R.E. Shope, associate editors. Raven Press, New York, N.Y., 1985.

Co-chief Editor, *Fundamental Virology*, B. Fields, co-chief editor; R.M. Chanock, J.L. Melnick, B. Roizman and R.E. Shope, associate editors. Raven Press, New York, N.Y., 1986.

Co-chief Editor, *Virology*, 2nd edition, B. Fields, co-chief editor; R.M. Chanock, M. Hirsch, J.L. Melnick, T. Monath and B. Roizman, associate editors. Raven Press, New York, N.Y., 1990.

Co-chief Editor, *Fundamental Virology*, 2nd ed., B. Fields, co-chief editor, R.M. Chanock, M.S. Hirsch, J.L. Melnick, T.P. Monath, B. Roizman, associate editors. Raven Press, New York, N.Y., 1991.

Co-chief Editor, *Virology*, 3rd ed., B.N. Fields, P.M. Howley, co-editors, Lippincott-Raven Publishers, Philadelphia, P.A., 1996.

Member, Journal of Virology editorial board, 1986-1997.

Member, Virology editorial board, 1986-1996.

Editor, *Virology*, 1996-present.

### Fellowships and Awards

National Science Foundation predoctoral fellowship, 1972-1975.

Jane Coffin Childs postdoctoral fellowship, 1976-1978.

Leukemia Society of America Special Fellowship, 1978-1980.

Cancer Research Scholar of American Cancer Society, Massachusetts Division, 1981-1984.

Faculty Research Award, American Cancer Society, 1984-1989.

MERIT Award, National Cancer Institute, NIH, 1988-1996.

Distinguished Lecturer in Molecular Genetics, Louisiana State University, School of Veterinary Medicine, 1994

#### National and International Committees

Member, Clinical Sciences Study Section, National Institutes of Health, 1985-1989.

Co-organizer, Fourteenth International Herpesvirus Workshop, Nyborg, Denmark, 1989.

Member, Virology Study Section, National Institutes of Health, 1990-1994.

Member, American Type Culture Collection Herpesvirus Advisory Committee, 1991.

Co-organizer, Seventeenth International Herpesvirus Workshop, Edinburgh, Scotland. 1992.

Member, Virology Study Section, NIH, 1997-2001.

Member, Biomedical Research Committee, The Medical Foundation, 1997-present.

#### Teaching

Course director and lecturer for Microbiology 101b, "Animal Virology," 1981-1985.

Lecturer and lab instructor for Microbiology 701, "Pathophysiology of Infectious Disease," 1982, 1984-1989.

Lecturer for Microbiology and Molecular Genetics Continuing Education Course, 1983-1987.

Course director for Virology 201, "Seminar in Animal Virology," 1986.

Course director and lecturer for Virology 101, "Animal Virology," 1986.

Course co-director and lecturer for Microbiology 201, "Molecular Biology of Eukaryotic Cells & Viruses," 1988.

Course director and lecturer for Virology 200, "Animal Virology," 1990-1991.

Lecturer for Virology 200, "Animal Virology," 1992-1994, 1996-1997.

Lecturer and discussion leader for Virology 201, "Virology Seminar," 1994.

Tutor for Harvard Medical School medical student course, "Identity, Microbes and Defense," 1995.

Course director and lecturer for Harvard Medical School medical student course, "Immunology, Microbiology and Infectious Disease," 1997-1998.

### Patents

1. Patent application: Serial number 922,912. "Herpesvirus Vaccines." Filed July 31, 1992.
2. Patent application: Serial number 08/179,106. "Herpesvirus Replication-defective Mutants." Filed January 10, 1994 as a continuation-in-part of 922,912.
3. Patent application: Serial number 08/278,601. "Herpesvirus Replication-defective Mutants." Filed July 21, 1994 as a continuation-in-part of 08/179,106.
4. Patent application: Serial number 08/393,879. "Mucosal and Systemic Co-Immunization Against Mucosal Infections". D.M. Knipe, X. DaCosta and L.A. Morrison, inventors. Filed February 24, 1995.
5. "Method of Identifying Compounds Which Modulate Herpesvirus Infection". D.M. Knipe, K. Xia and N.A. DeLuca. Filed January 5, 1996.

## Publications

1. Goldman, R.D. and D.M. Knipe. 1973. Functions of cytoplasmic fibers in non-muscle cell motility. Cold Spring Harbor Symposium on Quantitative Biology 37:523-534.
2. Rose, J. and D.M. Knipe. 1975. Nucleotide sequence complexities, molecular weights, and poly (A) content of the vesicular stomatitis virus mRNA species. Journal of Virology 15:994-1003.
3. Knipe, D.M., J. Rose and H. Lodish. 1975. Translation of individual species of vesicular stomatitis viral mRNA. Journal of Virology 15:1004-1011.
4. Knipe, D.M., H.F. Lodish and D. Baltimore. 1977. Localization of two cellular forms of the vesicular stomatitis viral glycoprotein. Journal of Virology 21:1121-1127.
5. Knipe, D.M., D. Baltimore and H.F. Lodish. 1977. Separate pathways of maturation of the major structural proteins of vesicular stomatitis virus. Journal of Virology 21:1128-1139.
6. Knipe, D.M., H.F. Lodish and D. Baltimore. 1977. Analysis of the defects of temperature-sensitive mutants of vesicular stomatitis virus: Intracellular degradation of specific viral proteins. Journal of Virology 21:1140-1148.
7. Knipe, D.M., D. Baltimore and H.F. Lodish. 1977. Maturation of viral proteins in cells infected with temperature-sensitive mutants of vesicular stomatitis virus. Journal of Virology 21:1149-1158.
8. Katz, F., J.E. Rothman, D.M. Knipe and H.F. Lodish. 1977. Membrane assembly: Synthesis and intracellular processing of the vesicular stomatitis viral glycoprotein. Journal of Supramolecular Structure 7:353-370.
9. Katz, F., J.E. Rothman, D.M. Knipe and H.F. Lodish. 1977. Membrane assembly: Synthesis and intracellular processing of the vesicular stomatitis viral glycoprotein. In: *Cell Surface Carbohydrates and Biological Recognition*. Progress in Clinical and Biological Research. Vol. 23. ICN-UCLA Symposia on Molecular Biology, V.T. Marchesi, V. Ginsberg, P. Robbins, E.F. Fox, eds. Liss, Inc., New York.



10. Lodish, H.F., F. Katz, J.E. Rothman, and D.M. Knipe. 1977. Membrane assembly: Synthesis and intracellular processing of the vesicular stomatitis viral glycoprotein. In: *Molecular Basis of Cell-Cell Interaction*. Birth Defects: March of Dimes Original Article Series, Vol. XIV, No. 2, pp. 155-175. R.A. Lerner and D. Bergsma, eds. Liss, Inc., New York.
11. Knipe, D.M., W.T. Ruyechan, B. Roizman and I.W. Halliburton. 1978. Molecular genetics of herpes simplex virus: Demonstration of regions of obligatory and non-obligatory identity within diploid regions of the genome by sequence replacement and insertion. *Proc. Natl. Acad. Sci. U.S.A.* 75:3896-3900.
12. Roizman, B., R.J. Jacob, D.M. Knipe, L.S. Morse, and W.T. Ruyechan. 1978. On the structure, functional equivalence and replication of the four arrangements of herpes simplex virus DNA. *Cold Spring Harbor Symposium on Quantitative Biology* 43:809-826.
13. Ruyechan, W.T., L.S. Morse, D.M. Knipe and B. Roizman. 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. *Journal of Virology* 29:677-697.
14. Knipe, D.M., W.T. Ruyechan and B. Roizman. 1979. Molecular genetics of herpes simplex virus. III. Fine mapping of a genetic locus determining resistance to phosphonoacetate by two methods of marker transfer. *Journal of Virology* 29:698-704.
15. Knipe, D.M., W.T. Ruyechan, R.W. Honess and B. Roizman. 1979. Molecular genetics of herpes simplex virus. IV. The terminal a sequences of the L and S components are obligatorily identical and constitute a part of a structural gene mapping predominantly in the S component. *Proc. Nat. Acad. Sci. U.S.A.* 76:4534-4538.
16. Knipe, D.M., W. Batterson and B. Roizman. 1980. A gene function of herpes simplex virus required for expression of all early viral genes. "Animal Virus Genetics," ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. XVIII, B. Fields, R. Jaenisch and C.F. Fox, eds. Academic Press, New York, N.Y. pp. 369-378.
17. Roizman, B., W. Batterson and D.M. Knipe. 1980. The potential significance of late herpes simplex virus functions expressed early in replication: The tale of a temperature-sensitive mutant. *Perspectives in Virology* 11:77-92.
18. Knipe, D.M., W. Batterson, C. Nosal, B. Roizman and A. Buchan. 1981. Molecular genetics of herpes simplex virus. VI. Characterization of a temperature-sensitive mutant defective in the expression of all early viral gene products. *Journal of Virology* 38:539-547.

19. Conley, A.J., D.M. Knipe, P.C. Jones and B. Roizman. 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. *Journal of Virology* 37:191-206.
20. Knipe, D.M. 1982. Cell growth transformation by herpes simplex virus. *Progress in Medical Virology* 28:114-144.
21. Knipe, D.M., and A.E. Spang. 1982. Definition of a series of stages in the association of two herpesviral proteins with the cell nucleus. *Journal of Virology* 43:314-324.
22. Knipe, D.M., M.P. Quinlan and A.E. Spang. 1982. Characterization of two conformational forms of the major DNA-binding protein encoded by herpes simplex virus 1. *Journal of Virology* 44:736-741.
23. Spang, A.E., P.J. Godowski and D.M. Knipe. 1983. Characterization of herpes simplex virus 2 temperature-sensitive mutants whose lesions map in or near the coding sequences for the major DNA-binding protein. *Journal of Virology* 45:332-342.
24. Quinlan, M.P. and D.M. Knipe. 1983. Nuclear localization of herpesviral proteins: Potential role of the cellular framework. *Molecular and Cellular Biology* 3:315-324.
25. Lee, C.K., and D.M. Knipe. 1983. Thermolabile in vivo DNA-binding activity associated with a protein encoded by mutants of herpes simplex virus type 1. *Journal of Virology* 46:909-919.
26. Godowski, P.J. and D.M. Knipe. 1983. Mutations in the major DNA-binding protein gene of herpes simplex virus type 1 result in increased levels of viral gene expression. *Journal of Virology* 47:478-486.
27. Rafield, L.F. and D.M. Knipe. 1984. Characterization of the major mRNAs transcribed from the genes for glycoprotein gB and ICP8 DNA-binding protein of herpes simplex virus type 1. *Journal of Virology* 49:960-969.
28. Quinlan, M.P., L.B. Chen and D.M. Knipe. 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell* 36:857-868.

29. Knipe, D.M. 1985. Molecular Genetics of Animal Viruses. In: *Virology*, B.N. Fields, ed. Raven Press, New York, N.Y. pp. 129-143.
30. Quinlan, M.P. and D.M. Knipe. 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. *Molecular and Cellular Biology* 5:957-963.
31. Quinlan, M.P. and D.M. Knipe. 1985. A genetic test for expression of a functional herpes simplex virus DNA-binding protein from a transfected plasmid. *Journal of Virology* 54:619-622.
32. Lee, C.K. and D.M. Knipe. 1985. An immunoassay for the study of DNA-binding activities of the herpes simplex virus protein ICP8. *Journal of Virology* 54:731-738.
33. Godowski, P.J. and D.M. Knipe. 1985. Identification of a herpes simplex virus function that represses late gene expression from parental viral genomes. *Journal of Virology* 55:357-365.
34. Foster, C.S., R. Wetzig, D.M. Knipe and M.I. Greene. 1985. Genetic influence from chromosome 12 on murine susceptibility to herpes simplex keratitis. In: *Herpetoc Eye Diseases*, Dr. W. Junk Publishers, Dordrecht/Boston/Lancaster.
35. Godowski, P.J. and D.M. Knipe. 1986. Transcriptional control of herpesvirus gene expression: Gene functions required for positive and negative regulation. *Proc. Natl. Acad. Sci. U.S.A.* 83:256-260.
36. Knipe, D.M. and J.L. Smith. 1986. A mutant herpesvirus protein leads to a block in nuclear localization of other viral proteins. *Molecular and Cellular Biology* 6:2371-2381.
37. Foster, C.S., Y. Tsai, J.G. Monroe, R. Campbell, M. Cestari, R. Wetzig, D.M. Knipe and M.I. Greene. 1986. Genetic studies on murine susceptibility to herpes simplex keratitis. *Clinical Immunology and Immunopathology* 40:313-325.
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8 PM Cells bank ready to harvest, but let go till the  
morrow.

For each stock: 406R & 504R

Shake off  
Into 2<sup>nd</sup> 200 ml bottle.  
10', 1.5k

Resuspend in 20 ml 10 ml supe + 10 ml milk.

Freeze @ -70

Thaw 2x

Sonicate 3x30" @ salting 4.

Aliquot to ~40 vials. "M28 n406R".

"M28 n504R".

Freeze @ -70°.

Titre on V27, Vero

V27's just confluent  
Vero nice

	V27						
n406R	-3	-4	-5	-6	-7	-8	
n504R	-3	-4	-5	-6	-7	-8	
KOS	-3	-4	-5	-6	-7	-8	

Vero							
-1	-2	-3	-4	-5	-6		
-1	-2	-3	-4	-5	-6		
	-3	-4	-5	-6	-7	-8	

0.2  
1.5  
etc.

6 well trays  
1 ml / well

V27's are light;  
add, or overlay w/  
serum + Ig  
fraction in  
med + 10% serum + Ig  
fraction in  
use 0.15 ml med  
75% is 0.1 ml in  
then uses 2x

	-1	-2	-3	-4	-5	-6	-7	-8
n406R - V27			>>	>>	>>	>>	31	2
n504R - V27			>>	>>	>>	>>	32	3
K05 - V27			>>	>>	>>	>>	54	11
n406R - Vero	k	k	Ø	Ø	Ø	Ø		
n504R - Vero	k	k	Ø	Ø	Ø	Ø		
K05 - Vero			>>	>>	>>	>>	105	23

k = killed monkeys

Titers for mnts V27:

Vero

n406R	$3 \times 10^8$	$< 1 \times 10^3$
n504R	$4 \times 10^8$	$< 1 \times 10^3$

APPENDIX D

Stock of d301.a

(1) 1.9-T-150 S-2 P10  $10^7$  cells / T-150

(2) d301A-1  $1.2 \times 10^7$

(3)  $0.01 \text{ hr} \times 4 \text{ floaks} \times 10^7 = 4 \times 10^5$

$$4 \times 10^5 / 1.2 \times 10^7 = 33 \lambda / 40 \text{ ml}$$

10 ml / T150 37°C / hr  
Suck off, add 45 ml 100% 0/g  
37°C

AM Harvest add 12 ml Dipep, 6 ml milk, - 8.

Sonicated. aliquots

" d301.a "

vero  $10^{-2}$   $10^{-3}$   $10^{-7}$   $10^{-8}$   
O (many cells) O

S-2 P17 85 6

$$85 \times 2 \times 10^7 = 1.7 \times 10^9$$

vid glück! All virus received from Dr. David Knipe.

① Injection time with:

① ICP<sub>8</sub> 10<sup>6</sup> pfu n = 8 Balb

ICP<sub>8</sub> stock d301 (= received from Dr. David Knipe's Lab (Kay) on day

1.7 10<sup>9</sup> pfu / cc ~~17~~ 17 10<sup>8</sup> pfu / cc.  
Need 10<sup>6</sup> pfu / cc.

so do: a 1: 1700 Dilution  
that means: 100  $\lambda$  in 170000 = 170 cc  
(virus stock)

or: 100  $\lambda$  in 85 cc PBS  
and injection of 5 cc

② ICP<sub>27</sub>: (n 504 R)  $4 \times 10^8$  pfu/cc  
n = 8

Need 10<sup>6</sup> pfu / cc: Balb mice.

So do: a 1: 4 10<sup>2</sup> Dilution  
that means 100  $\lambda$  in 40000 = 40 cc. PBS  
(virus)

or 100  $\lambda$  in 20 cc  
and inject 0.5 cc.

③ ICL<sub>4</sub>: (received from Dr. Neal de Luca)

5.5 10<sup>9</sup> pfu / cc.  
Need 100  $\lambda$  then do a  
1: 5.5 10<sup>2</sup> = 550 Dilution

Experiment:

(1) Bell mice / c Antac ♀.  
5-7 weeks by

Challenged			$\bar{c}$	
$10^6$ pfu	HSV	ICP <sub>4</sub>	$n = 8$	(2)
$10^6$ pfu	HSV	ICP <sub>8</sub>	$n = 8$	(2)
$10^6$ pfu	HSV	ICP <sub>27</sub>	$n = 8$	(3)
and			$n = 9$	(4)
PBS	as	Control		

1. Bleeding
2. Bleeding

Challenge  $\bar{c}$   $10^8$  pfu

HSV - mp.  
mortality:  
in 10 days

(2) (ICP<sub>4</sub>)

2 died / from 6.  
(2 for proliferation -  
array) total

(2) (ICP<sub>8</sub>)

0 died.

(3) (ICP<sub>27</sub>)

0 died.

(4) 0 died from 9. (Control)

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